TRANSFORMATION OF POTATO TUBER DISCS USING AGROBACTERIUM

II. POTATO TRANSFORMATION

Materials needed:

Sterile Microtubers

Fresh agrobacterium culture (to be diluted 1:10 in sterile MS below for transformation)

Forceps and scalpel

Vessels for bacterial incubation and subsequent rinse (can be same vessel)

Liquid MS pH 5.4 (sterilized and stored in 50-100ml alloquots up to12 months)

Sterile water

A sterile surface to cut tubers on (large petri dish)

Sterile filter paper

Plates (falcon 1029 or 1005) of Co-cultivation media

Grow up Agro:

- 1. Inoculate 10 ml of LB (+ Gm 20 or +Km 50 [for plasmid selection], + Strep [for Agrobacterium selection]) with transformed Agro. Grow up at 28°C overnight.
- 2. The following morning, dilute the overnight culture 1:10 into fresh Lb + antibiotics + **100µm acetosyringone (final conc.)**. Grow up culture for approx. 4 hours (should be at log phase).

Infect Potatoes:

- 1. Dilute fresh culture 1:10 with sterile MS pH 5.4 (depending on agro strain).
- 2. Remove tuber culture from sterile culture vessels and cut away debris--transfer intact minitubers to a clean cutting dish.
- 3. Add a small amount of sterile water to one side of the dish. Slice minitubers into 1-2mm thick slices. Push cut slices into water to prevent suberization while cutting remaining material.
- 4. Transfer cut disks to diluted culture, or pour off water and add diluted culture to cutting dish. Incubate in agro/MS at room temp for 10-15 minute. Occasionally stir or swirl slices while incubating in agro/MS, if desired.
- 5. Pour off agro solution and replace with sterile water or MS. Swirl slices in water (or MS) for 5 min, then remove slices and blot dry on sterile filter paper.
- 6. Gently place disks, large side down, on Cocultivation med for 2-3 days (23°C, 16 hr light photoperiod). The largest cut surface of the disk should make full contact with, yet not break the surface of, the media. Disks can be placed fairly densely on plates (0.5 cm apart).
- 7. After 2-3 days of co-cultivation, a moderate (visible, but not excessive) amount of bacterial growth should be evident <u>on the media</u> around the base of each disk. The surfaces not in contact with media should begin suberizing and drying. Following incubation on Cocult. med. transfer to stage one media.

III. REGENERATION

- Day 1: Transfer the tuber slices to Stage 1 medium in Falcon 1005 plates. Disks should be placed 8-10 per plate.
- Day 7, 14, 21 Transfer to fresh Stage I medium. Transfer to Stage II if callous growth is excessive. Throughout the first month of transfer, cut shoots that generate from the disk and discard. Most of these first shoots are not transgenic--most are growing from 'eyes'.

 Grooming and pruning the disk and callus seems to promote better health of subsequent tissue.

Day 28 and beyond: Continue to remove and discard shoots that are not growing from callus. There may not be many shoots at this time.

- Transfer disks to Stage 2 medium as usual. Green callus should begin to grow on the surface in contact with the media, mostly from the expanding cells in the periderm. White callus or callus that is not in contact with media (growing from top of tuber slice) is not likely to produce strong transgenic shoots and may be excised. As callus and shoots develop you may want to transfer the tissue to stage 2 in magenta boxes.
- As shoots start to emerge from the **callus**, cut them off and transfer to Stage 3 medium. Leave the disks/callus on Stage 2 (or transfer to fresh stage 2 media) to form more shoots. If green callus growth is very abundant and the original tuber tissue begins to look unhealthy, it is O.K. to discard original tissue and culture the callus alone.
- Keep track of which disks/callus the various shoots come from by assigning a number to each disk that forms callus. Each disk/callus may be considered one clone—all shoots from the same callus will be assumed to be the same clone. If callus growth begins in two discrete areas with ample area of non-growth between, the disk can be cut in half and each half may be considered a unique transformation event and, thus, a unique clone. This is up to the discretion of the researcher.
- When shoots grow and form roots on Stage 3, slice the shoot portion into nodal segments with a meristem in each segment and transfer to Stage 3 medium again. This increases the number of shoots and insures that the shoots were real transformants. It is also the time to do a Northern analysis to make absolutely sure that the plants you have are really real transformants. BE EVER VIGILANT! WATCH FOR SIGNS OF BACTERIAL CONTAMINATION. Once you have healthy roots on the stage 3 plants, you can transfer them to vermiculite.

IV. TRANSFERING PLANTS FROM CULTURE TO SOIL

- 1. Fill an appropriate amount of 2.5 inch pots with medium vermiculite. Completely saturate vermiculite with dilute miracle grow or 1/2 strength MS--it may take a while for the vermiculite to absorb the liquid. The vermiculite can be sterilized, but clean, unused vermiculite will do fine.
- 2. Gently break up agar around plantlet and remove the plant from media and place in a small dish of dilute miracle grow, water, or 1/2 strength MS. Gently wash and try and remove large pieces of agar from the roots. It is not bad to leave agar on the roots, but too much may promote fungus growth once planted in vermiculite.
- 3. Next, poke a hole in the vermiculite, place plantlet in hole and gently press vermiculite into hole and around the base of plantlet. Place the pots in trays and/or flats that can be sealed to retain moisture and allow light in. Initially, keep light low and moisture high. Once plant is acclimated (4-7 days), allow more light and air flow (lift lid of tray)-- Beware of desiccation! Vermiculite must be kept moist. Plant up to a bigger pot with commercial potting soil when appropriate--usually 2 plants per large pot works well. Be careful when exposing young plants to full light or intense heat--shielding from bright, direct light may be advised for the first day or two in big pots.

V. MEDIA Autoclave times are for 500ml. Less media may take less time to autoclave. Autoclaving aliquots > 500ml is not recommended. MS Salts = Caisson Labs #MSP001 = Sigma #5524 Phytotechnology Labs #M524 LS Salts = Caisson Labs #LSP001 = Sigma #6899 A. Shoot Multiplication Medium (1 Liter) {Blue dot color code} LS Salts 4.4 grams Sucrose 30.0 grams MSMO vitamin stock 1ml of stock (freezer) Adjust pH to 5.6 Phytagel (GelRite) 2.0
Autoclave for 20 minutes. Cool to 55°C.
Dispense to sterile Magenta boxes 2.0 grams Alternatively---melt in microwave, pour into tall boxes, and autoclave filled boxes B. Tuberization Medium (1 liter) {Salmon dot color code} LS Salts 4.4 grams Sucrose 60.0 grams Kinetin (cytokinin) Adjust pH to 5.6 2.5 ml of 1mg/ml stock Phytagel (GelRite)
Microwave to melt Phytagel. 2.0 grams Dispense into short boxes. Autoclave filled boxes for 15 minutes. C. Stage I Medium (1 Liter) {Yellow dot color code} MS Salts 4.3 grams Sucrose 20.0 grams Adjust pH to 5.6 Phytagel (Gelrite) 2.0 grams Autoclave 20 minutes. Cool to 55°C. Move to hood and add. Carbenicillin 10 ml of 50 mg/ml stock. (500µg/ml) Kanamycin 2 ml of 50 mg/ml stock (100µg/ml) Gamborg's Vitamin Stock (freezer) 1.0 ml Zeatin Riboside (freezer) IAA-Aspartic Acid (freezer) 1.0 ml of 10mM stock 0.1 ml of 3mM stock Dispense into Falcon 1005 petri dishes (or short boxes). D. Stage II Medium (1 Liter) (Orange dot color code) 4.4 grams (contains inositol & thiamine) LS Salts Sucrose 20.0 grams Adjust pH to 5.6
Add Phytagel (GelRite)
Autoclave 20 minutes. Cool to 55°C. Move to hood.
Aseptically, add: 2.0 grams Gibberellic Acid Zeatin Riboside (freezer) 1.0 ml stock (10 mg/ml in EtOH) 1.0 ml 10 mM sterile stock Kanamycin (freezer) 2 ml of 50 mg/ml sterile stock Carbenicillin (freezer) 10 ml of 50 mg/ml sterile stock MSMO Vitamin Stock (freezer) 1ml (OPTIONAL in emergency, add Cefotaxime as requested by researcher)
Dispense to sterile Magenta boxes with membrane lids.. E. Stage III Medium (1 Liter) Kanamycin Selection (Green dot color code) LS Salts 4.4 grams 30.0 grams Sucrose Adjust pH to 5.6 Add Phytagel (GelRite) 2.0 gram
Autoclave 20 minutes. Cool to 55°C. Move to hood.
Add MSMO Vitamin Stock (freezer) 1.0 ml 2.0 grams 4 ml of 50 mg/ml stock (200 µg/ml final) Add Kanamycin Add Carbenicillin 10 ml of 50 mg/ml stock. (500 µg/ml final) (OPTIONAL in emergency, add Cefotaxime as requested by researcher) Dispense to sterile Magenta boxes. F. Co-cultivation Medium (1 Liter) {Yellow dot color code} MS Salts 4.3 grams 20.0 grams Sucrose

Adjust pH to 5.4 pH is important for vir induction. Phytagel (Gelrite) 2.0 grams Autoclave 20 minutes. Cool to 55°C. Move to hood and add. Gamborg's Vitamin Stock (freezer) 1.0 ml... Zeatin Riboside (freezer) 1.0 ml of 10mM stock IAA-Aspartic Acid (freezer) 0.1 ml of 3mM stock Dispense into Falcon 1005 petri dishes (or short boxes).

G. Vitamin and Hormone Stocks: Vitamins can be autoclaved with little degradation. Hormones should not be autoclaved in media.

3. Zeatin Riboside (freezer) 10mM stock 50 mg + 14.22 ml 0.1N NaOH. Filter sterilize and freeze aliquots at -20°C

4. IAA-Aspartic Acid (freezer) 3mM stock 0.871 mg/ml 0.5N NaOH. Filter sterilize and freeze aliquots at -20°C

5. Kinetin: 1mg/ml stock

Purchase 1mg/ml liquid from sigma (K3253) and dispense to sterile tubes. (Can be autoclaved in media with some loss of activity)

6. Gibberellic Acid: 10mg/ml stock

Dissolve 10mg/ml in 95-100% Ethanol. Aliquot and store at -20°C

H. Antibiotic stocks:

Filter all water soluble antibiotics w/0.22 micron filters, aseptically transfer to sterile tubes and store at -20°C

Conc. in Media Stock 50mg/ml Carbenicillin 500µg/ml 100 (stg1,2)-200(stg3) µg/ml 200-400µg/ml researcher's discretion 50 mg/ml 100mg/ml Kanamycin Cefotaxime Gentamycin 20mg/ml 20µg/ml

LS Salts	A. Shoot Multiplication Medium (1 Liter)		250ml	500ml	750ml
Sucrose	LS Salts	4.4 grams	1.1	2.2	3.3
MSMO vitamin stock	Sucrose	30.0 grams	7.5	15.0	22.5
Adjust pH to 5.6 Autoclave for 20 minutes. Cool to 55°C. Dispense to sterile Magneta boxes Alternatively—melt in microwave, pour into tall boxes, and autoclave filled boxes Alternatively—melt in microwave, pour into tall boxes, and autoclave filled boxes Alternatively—melt in microwave, pour into tall boxes, and autoclave filled boxes 3. Tuberization Medium (1 liter) LS Salts. 4.4 grams. 1.1. 2.2. 3.3 Sucrose. 6.0.0 grams. 6.0.0 grams. 6.0.0,625ml 1.25ml 1.25ml 1.275ml Phylapel (GelRite). 2.0 grams. 0.5. 1.0. 1.5 Microwave to melt Phylapel. Dispense into short boxes. Autoclave filled boxes for 15 minutes. 2. Stage I Medium (1 Liter). 3. Sucrose. 2.0 grams. 4.3 grams. 4.3 grams. 5.0. 3. Sucrose. 2.0 grams. 5.0. 4.10. 4.15 Adjust pH to 5.6 Aphylapel (GelRite). 2.0 grams. 3. Sucrose. 3. Sucrose. 3. O.5. 4.10. 4.15 Autoclave 20 minutes. Cool to 55°C. Move to hood and add displayed an	MSMO vitamin stock	1ml of stock (freezer)	0.25	0.5	0.75
Phytage (GelRite)		11111 Of 3tock (11cc2ci)	0.20		
Autoclave for 20 minutes. Cool to 55°C. Dispense to sterile Magnetia boxes Alternatively—melt in microwave, pour into tall boxes, and autoclave filled boxes 3. Tuberization Medium (1 liter)	Phytagal (CalPita)	2.0 grame	0.5	1.0	15
Dispense to sterile Magenta boxes Alternativelymelt in microwave, pour into tall boxes, and autoclave filled boxes	Autodovo for 20 minutos Cool to 5	2.0 yrairis	0.3		1.3
Alternativelymelt in microwave, pour into tall boxes, and autoclave filled boxes 3. Tuberization Medium (1 liter)					
3. Tuberization Medium (1 liter)	Dispense to sterile Magenta boxes		90 1 1		
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Microwave to melt Phytagel. Dispense into short boxes. Autoclave filled boxes for 15 minutes. 2. Stage I Medium (1 Liter)	Adjust pH to 5.6				
Dispense into short boxes. Autoclave filled boxes for 15 minutes. 2. Stage I Medium (1 Liter)		2.0 grams	0.5	1.0	1.5
Autoclave filled boxes for 15 minutes. 2. Stage I Medium (1 Liter)					
C. Stage I Medium (1 Liter)					
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MS Salts.	J. Stage I Medium (1 Liter)		250ml	500ml	750ml
Sucrose	MS Salts	4.3 grams	1.08	2.15	3.0
Adjust pH to 5.6 Phytagel (Gelrite) 2.0 grams 0.5 1.0 1.5 Autoclave 20 minutes. Cool to 55°C. Move to hood and add. Carbenicillin 10 ml of 50 mg/ml stock 2.5 5.0 7.5 Kanamycin 2 ml of 50 mg/ml stock. 0.5 1.0 1.5 Gamborg's Vitamin Stock (freezer) 1.0 ml 0.25 0.5 0.75 Zeatin Riboside (freezer) 1.0 ml of 10mM stock. 0.25 0.5 0.75 IAA-Aspartic Acid (freezer) 0.1 ml of 3mM stock 0.025 0.05 0.075 Dispense into Falcon 1005 petri dishes (or short boxes). D. Stage II Medium (1 Liter) 2.0 grams 1.1 2.2 3.3 Sucrose 2.00 grams 1.1 2.2 3.3 Sucrose 2.00 grams 5.0 10.0 15.0 Adjust pH to 5.6 Add Phytagel (GelRite) 2.0 grams 0.5 1.0 1.5 Autoclave 20 minutes. Cool to 55°C. Move to hood. Asseptically, add; Gibberellic Acid 1.0 ml stock 0.25 0.5 0.75 Zeatin Riboside (freezer) 1.0 ml 10 mM stock 0.25 0.5 0.75 Kanamycin (freezer) 2 ml of 50 mg/ml stock 0.25 0.5 0.75 Zeatin Riboside (freezer) 1 ml of 50 mg/ml stock 0.25 0.5 0.75 MSMO Vitamin Stock (freezer) 10 ml of 50 mg/ml stock 0.25 0.5 0.75 Dispense to sterile Magenta boxes with membrane lids. Exage III Medium (1 Liter) Kanamycin Selection 250ml 5.0 1.5 Add Phytagel (GelRite) 2.0 grams 1.1 2.2 3.3 Sucrose 3.00 grams 1.1 2.2 3.3 Sucrose 3.00 grams 1.1 2.2 3.3 Sucrose 3.00 grams 3.1 3.1 3.1 3.3 Sucrose 3.00 grams 3.1 3.1 3.3 Sucrose 3.00 grams 3.1 3.1 3.3 Sucrose 3.00 grams 3.1 3.3 Sucrose 3.0	Sucrose	20.0 grams	5.0	10.0	15.0
Phytagel (Gelrite) 2.0 grams 0.5. 1.0 1.5 Autoclave 20 minutes. Cool to 55°C. Move to hood and add. Carbenicillin 10 ml of 50 mg/ml stock. 2.5. 5.0 7.5 Kanamycin 2 ml of 50 mg/ml stock. 0.5 1.0 1.5 Gamborg's Vitamin Stock (freezer) 1.0 ml 0.25 0.5 0.75 Zeatin Riboside (freezer) 1.0 ml 0.25 0.5 0.75 IAA-Aspartic Acid (freezer) 0.1 ml of 10mM stock 0.25 0.5 0.75 Dispense into Falcon 1005 petri dishes (or short boxes). D. Stage II Medium (1 Liter) 0.1 ml of 3mM stock 0.025 0.05 0.075 Dispense into Falcon 1005 petri dishes (or short boxes). D. Stage II Medium (1 Liter) 250ml 500ml 750ml LS Salts 4.4 grams 1.1 22 3.3 Sucrose 20.0 grams 5.0 110 15.0 Adjust pH to 5.6 Add Phytagel (GelRite) 2.0 grams 0.5 1.0 15.0 Add Phytagel (GelRite) 2.0 grams 0.5 1.0 1.5 Utoclave 20 minutes. Cool to 55°C. Move to hood. Aseptically, add: Gibberellic Acid 1.0 ml stock 0.25 0.5 0.75 Zeatin Riboside (freezer) 1.0 ml 10 mM stock 0.25 0.5 0.75 Kanamycin (freezer) 2 ml of 50 mg/ml stock (100µg/ml). 0.5 1.0 1.5 Carbenicillin (freezer) 10 ml of 50 mg/ml stock (100µg/ml). 0.5 1.0 1.5 Carbenicillin (freezer) 10 ml of 50 mg/ml stock 0.25 0.5 0.75 Dispense to sterile Magenta boxes with membrane lids. E. Stage III Medium (1 Liter) Kanamycin Selection 250ml 500ml 750ml LS Salts 4.4 grams 1.1 2.2 3.3 Sucrose 30.0 grams 7.5 15.0 2.5 Add Phytagel (GelRite) 2.0 grams 0.5 1.0 1.5 Add Carbenicillin (200 µg/ml inal) Add Carbenicillin (200 µg/ml inal) MS Salts 4.3 grams 1.08 2.5 5.0 7.5 OPTIONAL in emergency, add Cefotaxime as requested by researcher) Dispense to sterile Magenta boxes. C. Co-cultivation medium (1 Liter) Add Salts 4.3 grams 1.08 2.5 5.0 7.5 DAJD Phytagel (GelRite) 2.0 grams 1.0 0.25 5.0	Adjust pH to 5.6				
Autoclave 20 minutes. Cool to 55°C. Move to hood and add. Carbenicillin	Phytagel (Gelrite)	2.0 grams	0.5	1.0	1.5
Carbenicillin	Autoclave 20 minutes. Cool to 55°C	C. Move to hood and add.			-
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Samborg's Vitamin Stock (freezer)	Kanamycin	2 ml of 50 mg/ml stock	0.5	1 0	1.5
Alay	Gambora's Vitamin Stock (fr		0.25	0.5	0.75
IAA-Aspartic Acid (freezer)	Zoatin Pihosido (froezer)	1.0 ml of 10mM stock	0.25 0.25	0.5	0.75
Dispense into Falcon 1005 petri dishes (or short boxes). D. Stage II Medium (1 Liter)	Zealin Riboside (neezer)	1.0 IIII OI TOITIIVI SLOCK	0.23	0.3	0.75
Dispense into Falcon 1005 petri dishes (or short boxes). D. Stage II Medium (1 Liter)	IAA Aspartia Asid (franzor)	0.1 ml of 2mM stock	0.025	0.05	0.075
D. Stage II Medium (1 Liter)	Dianana into Falcan 1005 notri dia	has (ar short haves)	0.023		0.073
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LS Salts	D. Stage II Medium (1 Liter)		250ml	500ml	750ml
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Add Phytagel (GelRite)		20.0 grams	5.0	10.0	15.0
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Gibberellić Acid.		e to nood.			
Zeatin Riboside (freezer)		40 1 1	0.05	0.5	0.75
Kanamycin (freezer)					
Carbenicillin (freezer)	Zeatin Riboside (freezer)	1.0 ml 10 mM stock	0.25	0.5	0.75
MSMO Vitamin Stock (freezer)	Kanamycin (freezer)	2 ml of 50 mg/ml stock (100µg/m	l)0.5	1.0	1.5
MSMO Vitamin Stock (freezer)	Carbenicillin (freezer)	10 ml of 50 mg/ml stock	2.5	5.0	7.5
OPTIONAL in emergency, add Cefotaxime as requested by researcher) Dispense to sterile Magenta boxes with membrane lids. E. Stage III Medium (1 Liter) Kanamycin Selection	MSMO Vitamin Stock (freezer)	1ml	0.25	0.5	0.75
Dispense to sterile Magenta boxes with membrane lids. E. Stage III Medium (1 Liter) Kanamycin Selection	OPTIONAL in emergency, add Cefotaxim	e as requested by researcher)			
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LS Salts	E. Stage III Medium (1 Liter) Kanamycin Se	election	250ml	500ml	750ml
Sucrose	LS Salts	4.4 grams	1.1	2.2	3.3
Adjust pH to 5.6 Add Phytagel (GelRite)	Sucrose	30.0 grams	7.5	15.0	22.5
Add Phytagel (GelRite) 2.0 grams 0.5 1.0 1.5 Autoclave 20 minutes. Cool to 55°C. Move to hood. 0.25 0.5 0.75 Add MSMO Vitamin Mix (freezer) 1.0 ml 0.25 0.5 0.75 Add Kanamycin 4 ml of 50 mg/ml stock 1.0 2.0 3.0 (200 µg/ml final) 2.5 5.0 7.5 OPTIONAL in emergency, add Cefotaxime as requested by researcher) 0.5 5.0 7.5 Dispense to sterile Magenta boxes. 2.5 5.0 7.5 C-Co-cultivation medium (1 Liter) 2.5 5.0 3.0 MS Salts 4.3 grams 1.08 2.15 3.0 Sucrose 20.0 grams 5.0 10.0 15.0 Adjust pH to 5.4 Phytagel (Gelrite) 2.0 grams 0.5 1.0 1.5 Autoclave 20 minutes. Cool to 55°C. Move to hood and add. 0.5 0.5 0.75 Zeatin Riboside (freezer) 1.0 ml 0.25 0.5 0.75 IAA-Aspartic Acid (freezer) 0.1 ml of 10mM stock 0.25 0.5 0.075	Adjust pH to 5.6	•			
Autoclave 20 minutes. Cool to 55°C. Move to hood. Add MSMO Vitamin Mix (freezer)	Add Phytagel (GelRite)	2.0 grams	0.5	1.0	1.5
Add MSMO Vitamin Mix (freezer)	Autoclave 20 minutes Cool to 55°C Mov	e to hood			
Add Kanamycin	Add MSMO Vitamin Miv (freezer)	1 0 ml	0.25	0.5	0.75
(200 μg/ml final) Add Carbenicillin	Add Kanamycin	4 ml of 50 mg/ml etack	0.∠0		2 N
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Dispense to sterile Magenta boxes. C. Co-cultivation medium (1 Liter)	Add Carbonicillin	10 ml of 50 mg/ml stock	2.5	5.0	75
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C. Co-cultivation medium (1 Liter)	Dispense to starile Magazite	e as requested by researcher)			
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Phytagel (Gelrite) 2.0 grams 0.5 1.0 1.5 Autoclave 20 minutes. Cool to 55°C. Move to hood and add. 0.25 0.5 0.75 Gamborg's Vitamin Stock (freezer) 1.0 ml 0.25 0.5 0.75 Zeatin Riboside (freezer) 1.0 ml of 10mM stock 0.25 0.5 0.75 IAA-Aspartic Acid (freezer) 0.1 ml of 3mM stock 0.025 0.05 0.075		20.0 grams	5.0	10.0	15.0
Autoclave 20 minutes. Cool to 55°C. Move to hood and add. Gamborg's Vitamin Stock (freezer)1.0 ml	Adjust pH to 5.4				
Autoclave 20 minutes. Cool to 55°C. Move to hood and add. Gamborg's Vitamin Stock (freezer)1.0 ml	Phytagel (Gelrite)	2.0 grams	0.5	1.0	1.5
Gamborg's Vitamin Stock (freezer)	Autoclave 20 minutes Cool to 55°C Move	e to hood and add			
Zeatin Riboside (freezer)	Gamborg's Vitamin Stock (freezer)	1.0 ml	0.25	0.5	0.75
IAA-Aspartic Acid (freezer)0.1 ml of 3mM stock0.0250.05	Zeatin Riboside (freezer)	1.0 ml of 10mM stock	0.25	0.5	0.75
Nignanga into Eglaga 1005 patri diphag (ar short havea)	IAA-Aspartic Acid (freezer)	0.1 ml of 3mM stock	0.025	0.05	0.075
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